AD_____

Award Number: DAMD17-98-1-8560

TITLE: Inhibition of Prostate Tumor Cell Invasiveness by Chemically Modified Tetracyclines with Broad Spectrum Antiproteinase Activity

PRINCIPAL INVESTIGATOR: Sanford R. Simon, Ph.D.

CONTRACTING ORGANIZATION: State University of New York
Stony Brook, New York 11794-3366

REPORT DATE: September 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20000809 078

REPORT DOCUMENTATION PAGE		Form Approved OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE September 1999	3. REPORT TYPE AND DATES COVERED Annual (1 Sep 98 - 31 Aug 99)	
4. TITLE AND SUBTITLE Inhibition of Prostate Tumor Cell Invasiveness by Chemically Modified Tetracyclines with Broad Spectrum Antiproteinase Activity			**5. FUNDING NUMBERS** DAMD17-98-1-8560
6. AUTHOR(S) Sanford R. Simon, Ph.D.			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) State University of New York Stony Brook, New York 11794-3366 E-MAIL: ssimon@path.som.sunysb.edu			**8. PERFORMING ORGANIZATION REPORT NUMBER**
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**
11. SUPPLEMENTARY NOTES			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			**12b. DISTRIBUTION CODE**

13. ABSTRACT *(Maximum 200 Words)*

We have evaluated novel nonantimicrobial chemically modified tetracyclines (CMTs) for management of invasive prostate cancer, based on preliminary results showing that one such CMT (CMT-3) inhibits activities of matrix metalloproteinases (MMPs) as well as the serine proteinase human neutrophil elastase, reduces levels of MMPs released by cells in culture, and exhibits selective cytotoxicity towards the human prostate tumor cell line LNCaP but not towards normal prostate stromal cells in culture. In response to photoreactions in patients receiving CMT-3 in Phase I clinical trials at NCI, we have screened all new CMTs with UV-A exposed NIH 3T3 cells to ensure that their phototoxicities *in vitro* are no greater than that of doxycycline. We have also evaluated the new CMTs for dose-dependent inhibition of collagenolytic and gelatinolytic activities of MMP-8 and MMP-9, as well as neutrophil elastase activity. The cytotoxicities of the new CMTs have been investigated using multiple criteria including membrane permeability to the DNA-binding dye SYTO 17 and failure to reduce the tetrazolium salt MTS. In addition to LNCaP cells, we use R22 rat smooth muscle cells and normal human epithelial cells as targets. The most promising CMTs display preferential dose-dependent cytotoxicity towards tumor cells, and induce morphologic changes, including rounding, detachment, and loss of confluence which mark a departure from the invasive phenotype. We plan to test the new CMTs with explant cultures of resected prostate tumors for inhibition of proteinase activities and cytotoxicity.

14. SUBJECT TERMS Prostate Cancer, tetracyclines, proteinase inhibitors, matrix metallo-proteinases, elastase, tumor cytotoxicity, phototoxicity		15. NUMBER OF PAGES 34
		16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

signature Where copyrighted material is quoted, permission has been obtained to use such material.

signature Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

signature Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

<div style="text-align:right">

signature   9/29/99

PI - Signature   Date

</div>

Table of Contents

A. Introduction

This report covers progress made on the tasks outlined in the Statement of Work for this project on the use of chemically modified tetracyclines (CMTs) in management of prostate cancer. Those tasks include: 1) screening normal prostate stromal and epithelial cells, prostate tumor cell lines, and prostate tumor explants for expression of proteinases; 2) measuring degradative activity of the prostate tumor cells towards stromal substrates; 3) evaluating the cytotoxic activity of chemically modified tetracyclines towards normal and tumor-derived prostate cells; 4) and 5) determining the capacity of the chemically modified tetracyclines to inhibit degradative activity of normal and tumor-derived prostate cells; and 6) comparing new generation tetracycline derivatives to the then current lead candidate for development as a drug for use in management of prostate cancer. The progress on the different tasks reflects the areas of greatest interest expressed by the USAMRMC in its critique of the original proposal, and although no modifications to the Statement of Work were proposed in that critique, we have responded especially to the interest expressed by the Army in the development of new "second generation" chemically modified tetracyclines for use in the treatment of prostate cancer (Task 6).

At the outset, it should be noted that the National Cancer Institute has continued to pursue Phase I clinical trials on the use of the chemically modified tetracycline described in the original Statement of Work (CMT-3 or 6-demethyl-6-deoxy-4-de(dimethylamino)tetracycline). The criteria for admission of patients to this trial included failure on all other chemotherapies, and it was understood that the primary objective of the trial was to garner information relating to safety of the drug rather than efficacy. As a result of observations of patients participating in this trial, a significant incidence of dermatologic complications associated with phototoxicity of CMT-3 were noted. These dermatologic complications consisted primarily of sunburn-like lesions which were in all cases located on sites exposed to sunlight, including face, arms and hands. These reactions have not been deemed so serious as to suspend further trials of CMT-3, which are now being undertaken at a consortium of sites across the country on patients with a variety of tumors other than primary prostate cancer or its metastases. Nevertheless, we have responded to these results by accelerating our efforts to identify "new generation" CMTs which have significantly less phototoxicity than CMT-3 (hereafter referred to as CMT-300) while retaining a profile of inhibition of matrix metalloproteinases as well as the serine proteinase human neutrophil elastase. Additionally, we have sought to identify CMTs which exhibit some selective cytotoxicity *in vitro* towards human tumor cells, especially established human prostate tumor cell lines, while displaying relatively low cytotoxicity towards normal human cells in culture. These studies have all been undertaken with the cooperation of CollaGenex Pharmaceuticals, Inc, which has provided the CMT-300 for the NCI clinical trials as well as all the new CMTs for our investigative work.

B. Phototoxicity Screening of New CMTs

We have engaged the assistance of Drs. John Harbell and Hans Raabe at the Institute for In Vitro Sciences in Bethesda, MD to screen approximately thirty different tetracycline derivatives for phototoxicity to the NIH 3T3 line of murine fibroblasts, using a protocol which has become internationally accepted as a standard for phototoxicity. The chemically modified tetracyclines for these phototoxicity studies were all provided by CollaGenex Pharmaceuticals Inc. as part of the contribution originally described in our Cost Sharing Agreement. Our criteria for inclusion of new tetracycline derivatives for further study were that they exhibit not only less phototoxicity than CMT-300, but also less phototoxicity than doxycycline, a tetracycline which retains antibiotic activity and is known to trigger an incidence of photoreactions in patients which is significant but

is still regarded as acceptable by the FDA. This phototoxicity screen involves incubation of the 3T3 cells for 50 minutes with the test compound in the dark or exposed to 5 Joules/cm^2 UV-A light, and provides some limited information about acute cytotoxicity as well as phototoxicity. Phototoxicity was measured as the IC$_{50}$ for UV-exposed cells and was related to the cytotoxicity towards cells cultured in the dark to obtain a PhotoIrritancy Factor (PIF) or, for those compounds which displayed minimal cytotoxicity in the dark, was reported as a Mean PhotoEffect (MPE). The results are presented in Table B.1. A more detailed presentation of this work by Drs. Harbell and Raabe and their colleagues and by Elizabeth Roemer in our laboratory is in press [1]. As mentioned in the Introduction, we have arbitrarily disqualified CMTs for further consideration as potential drug candidates in this project if they show greater phototoxicity than doxycycline. The two most phototoxic CMTs identified in this screen have been CMT-300 and CMT-800 (formerly CMT-8), both of which are much more phototoxic than doxycycline. However, several derivatives of these two CMTs have phototoxicities equivalent to or less than that of doxycycline. Among the original CMTs, the 4-de(dimethylamino) derivative of minocycline, CMT-310 (formerly CMT-10) was found to be particularly nonphototoxic, as were two of its derivatives, CMT-1001 and CMT-1002.

Drs. Nungavaram Ramamurthy and Steven McClain at Stony Brook, working with Dr. Golub (Collaborating Investigator on this project), have confirmed that of the ten original CMTs, CMT-300 and CMT-800 (originally CMT-3 and CMT-8) display significantly greater phototoxicity than doxycycline in an *in vivo* model of hairless rats exposed to 50-125 joules UV-A light after receiving CMTs via oral gavage. In this model, the erythematous reaction to UV exposure was clearly a function not only of intrinsic phototoxicity of the compounds but also of the plasma levels of the different drugs which were delivered at the same oral doses. These limited *in vivo* studies also confirmed the *in vitro* results which indicated that irrespective of the mechanism of phototoxicity, the reaction is clearly dose dependent with respect to CMT concentration. We will continue to employ the *in vitro* phototoxicity screen at the Institute for In Vitro Sciences along with our other criteria described below to prioritize drug development efforts with new CMT derivatives.

C. Antielastase Activity of New CMTs

We have used several types of measurements to assess antielastase activity of new CMTs. The most straightforward assay involves the use of purified human neutrophil elastase, isolated from purulent sputum of patients with cystic fibrosis (Elastin Products Corp) or from human neutrophils (Athens Research and Technology), and the oligopeptide chromogenic anilide, methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide (MeOSAAPVPNA), as substrate. It will be recalled from our original proposal that neutrophil elastase activity, which is normally associated with inflammatory cells, has been detected in especially invasive human breast tumors; an elastase activity has also been reported in several Dunning rat prostate tumor cell lines, including MatLyLu. Using the purified enzyme and the oligopeptide substrate, Christy Mannino in our laboratory has showed that the CMTs are simple competitive inhibitors of elastase activity for which K_I values could be computed from analysis of Dixon plots. She has confirmed these K_I values for elastase using crude preparations of sputum from cystic fibrosis patients which contain proteolytic activity from matrix metallo-proteinases in addition to neutrophil elastase activity. The inhibition of elastase activity reflects hydrophobic interactions between the enzyme and the CMTs. In the presence of serum albumin, the inhibitory potency of these hydrophobic CMTs is diminished as they bind to the hydrophobic pocket of the plasma protein. Moreover, human pancreatic elastase, which has a lower affinity than the neutrophil enzyme for a variety of hydrophobic anionic inhibitors, is much less sensitive than neutrophil elastase to inhibition by the CMTs. The CMTs are inactive as inhibitors of pancreatic

trypsin or chymotrypsin. Furthermore, the trypsin-like serine proteinase known as TAT (Tumor Associated Trypsin) produced by COLO 205 human colon carcinoma cells in culture, which Ying Gu in our laboratory has shown is capable of activating both MMP-2 and MMP-9 *in vitro*, is not sensitive to inhibition by CMTs. Representative results of antielastase K_I determination for a number of the new generation CMTs are summarized in Table C.1. Some of these results have also been presented at the 1999 Annual Meeting of the Society for In Vitro Biology [2].

A few of the more promising CMTs have also been checked by Christy Mannino for their capacity to inhibit the degradation of insoluble elastin by sputum elastase activity. Further confirmatory criteria for antiproteolytic activity of the new CMTs have been based on measurements by Bantoo Sehgal in our laboratory of inhibition of degradation of a complete interstitial extracellular matrix (ECM) derived from R22 rat smooth muscle cells, using cystic fibrosis sputum or live human neutrophils as sources of proteolytic activity. We have previously shown that the degradation of this interstitial matrix by neutrophils or purulent secretions which are principally composed of neutrophil proteases is mediated primarily by neutrophil elastase rather than by matrix metalloproteases. These assays have confirmed the apparent order of potency of the CMTs as inhibitors of neutrophil elastase as estimated from K_I determinations in solution. Representative results for IC_{50} determinations of inhibition of ECM degradation by CMTs, using inflammatory cells and their products as sources of degradative activity, are provided in Table C.2. Some of these results have also been presented at the 1999 Annual Meeting of the Society for In Vitro Biology [3].

D. New CMTs as Inhibitors of Matrix Metalloproteinases

We have employed two types of assays to screen potency of inhibition of the proteolytic activity of matrix metalloproteinases (MMPs) by CMTs. One assay adapted by Wayne Bellucci in our laboratory makes use of a novel substrate for MMPs developed by Verheijen et al, [4] who employed genetic engineering methods to replace the normal plasmin-sensitive activation sequence in human prourokinase with an amino acid sequence which is not cleaved by serine proteinases but rather only by MMPs. MMP activity generates urokinase activity which is then detected by amidolysis of a chromogenic urokinase-specific oligopeptide p-nitroanilide. The advantage of this assay is that considerable amplification of the MMP proteolytic activity is achieved by using another enzyme as the substrate. In the commercially available form of this assay marketed by Amersham Pharmacia, the MMP to be assayed is first "captured" by an antibody liked to a 96-well microplate and then activated by an organomercurial (p-aminophenyl mercuric acetate, APMA) on the plate. After washing the excess reagent away, the activated MMP is incubated with the engineered prourokinase and the urokinase substrate in the presence or absence of a selected CMT in a kinetic microplate reader and the distinctive exponentially increasing rate of p-nitroaniline release is recorded for 4 hours. Wayne Bellucci has demonstrated that the CMTs do not inhibit the APMA-mediated activation of MMP-9 in this assay. Moreover, if the activated MMP-9 is then allowed to activate the engineered prourokinase for 4 hours prior to addition of the urokinase substrate and the CMTs, none of the tested CMTs inhibit the active urokinase generated in this fashion. Thus, in our hands, the CMTs we have tested specifically inhibit the MMP-mediated activation of an engineered prourokinase which is achieved by clipping an MMP-specific cleavage sequence. These results have been presented at the 1999 Annual Meeting of the Society for In Vitro Biology [5] and are included in the data in Table D.1. At this time, we have only tested inhibition of MMP-9 proteolytic activity in this assay, but we will proceed to evaluate inhibition of MMP-2 activity as well, since its capacity to cleave the engineered urokinase is at least as great as that of MMP-9. These two gelatinolytic MMPs are produced in varying ratios by a number of tumor cell lines. For example, as demonstrated

7

by Ying Gu in our laboratory using gelatin zymography, COLO 205 colon carcinoma cells produce predominantly MMP-2, while MDA-MB-231 breast tumor cells produce predominantly MMP-9. Wayne Bellucci has very recently identified MMP-9 as the predominant gelatinolytic MMP released by LNCaP prostate tumor cells, although lesser levels of MMP-2 can also be detected on gelatin zymograms of LNCaP culture supernatants. Ying Gu has shown that CMT-300 inhibits the gelatinolytic activity of both these MMPs when it is included in the incubation medium used to develop the zymograms. Moreover, when the supernatant medium from COLO 205 cells or the E-10 subclone of MDA-MB-231 cells (engineered to overexpress MMP-9) was incubated with the R22 rat smooth muscle cell-derived ECM, both supernatants were capable of degrading the matrix after activation of the MMPs by TAT. (This TAT-mediated activation takes place spontaneously during culture of the COLO 205 cells but requires an *in vitro* incubation step for the E-10 culture supernatants, since these breast cancer cells do not synthesize their own TAT.) This ECM degradation could be inhibited by addition of 1,10-phenanthroline, implicating the MMPs in the supernatants as the source of the degradative activity. Moreover, degradation by both supernatants could be inhibited by CMT-300 in a dose-dependent fashion.

In a second assay for MMP activity, Hsi-Ming Lee in Dr. Golub's laboratory has employed a system marketed by Chemicon Corp for measuring the collagenolytic activity of MMP-8. This MMP is synthesized predominantly by human neutrophils although its expression has been detected at low levels in some tumor cell lines. In this assay, the substrate is native type I collagen which has been substituted on some of its lysine residues with fluorescein. Collagenolytic activity is detected as liberation of trichloroacetic acid-soluble fluorescent collagen cleavage products. The collagenolytic assay has the advantage of employing a substrate which is closer to the "natural" MMP substrates, but collagenolysis cannot be followed continuously. A modification of the assay using type IV collagen with MMP-2 or MMP-9 should permit us to compare results with the prourokinase-based assays. The most recent results we have obtained for inhibition of MMPs by the new generation CMTs are reported in Table D.1. Since the concentrations of the substrates used in the inhibition assays for MMP-8 and MMP-9 activity are different, it is difficult to compare the potencies of the different CMTs towards these two target MMPs, but there are relatively few cases in which the order of CMT potency is inconsistent.

In the course of her studies on COLO 205 cells and the E-10 clone of MDA-MB-231 cells, Ying Gu has demonstrated convincingly that CMT-300 not only inhibits MMP enzymatic activity but also reduces the levels of MMP-2 and MMP-9 in the supernatant medium of cultures which have been maintained for 48 hours in the presence of the tetracycline [6]. As discussed in the following section, the mechanism for reduced levels of MMP-2 in cultures of COLO 205 cells maintained in the presence of CMT-300 may reflect in part the cytotoxic effect of relatively low levels of the tetracycline (10-20 μM) on this cell line, but the comparable effect on levels of MMP-9 released by MDA-MB-231 cells is achieved at the same 10 μM and 20 μM levels of CMT-300 which are not cytotoxic to this cell line. At this time we have not yet determined the effects of the different new generation CMTs on levels of the proteinases produced by normal or tumor-derived prostate cells in culture. However, Mansi Kothari in our laboratory has obtained preliminary data which suggests that subcytotoxic levels of several of the CMTs have no significant effect on levels of the serine proteinase PSA (Prostate Specific Antigen) released by LNCaP cells in culture.

E. Cytotoxicity of the New CMTs

As described in the original proposal, CMT-300 appears to be especially cytotoxic towards the human prostate cancer cell line LNCaP. This cytotoxicity, which is dose dependent, can be

observed after exposure periods as short as a few hours. Another human prostate cancer cell line, PC-3, is sensitive to CMT-300 as well, but cytotoxicity is not apparent until 48-72 hours have elapsed. A third prostate cancer cell line, DU-145, is quite resistant to CMT-300 even after 72 hours. Ying Gu has demonstrated that CMT-300 also shows variable cytotoxicity towards other cancer cell lines: COLO-205 cells are quite sensitive to this derivative, with an IC_{50} on the order of 10-20 μM after 48 hours of exposure, whereas the E-10 engineered clone of MDA-MB-231 cells, which overexpresses MMP-9, is resistant to 50 μM CMT-300. Criteria for cytotoxicity in the studies on COLO-205 and MDA-MB-231 cells included reduction of the tetrazolium dye MTS to its formazan as well as release of the cytosolic enzyme lactic dehydrogenase into the supernatant.

Under the supervision of Elizabeth Roemer, who oversees all our cell culture studies, Stephanie Boumakis in our laboratory has undertaken a comprehensive study of the effects of the CMTs on the capacity of the LNCaP cell line to exclude a nucleic acid-specific fluorescent dye, SYTO-17, from the nucleus. The cells were maintained in 48 well microplates on which an interstitial ECM from R22 rat smooth muscle cells had been previously deposited as a substratum. Cells were maintained in the presence of the CMTs for 24 or 48 hours prior to incubation with the SYTO 17 dye. Figures E.1 through E.3 illustrate the response of the LNCaP cells to different doses of CMT-200 (a noncytotoxic CMT), CMT-300 (an especially cytotoxic CMT), and CMT-303 (a somewhat less cytotoxic CMT). Table E.1 summarizes the apparent IC_{50} values for the different CMTs after 24 and 48 hours of exposure, based on SYTO 17 permeability. These results were also presented at the 1999 Annual Meeting of the Society for In Vitro Biology [7]. The results are consistent with our earlier observations that CMT-300 can begin to induce cytotoxicity within hours, while other tetracycline derivatives, such as doxycycline, are cytotoxic only after 48 hours of incubation.

The progressive loss of membrane integrity which renders cells permeable to SYTO 17 can be correlated with a progressive loss of reductase activity which results in a failure to convert MTS to its formazan. Jacky Lie and Heather Sawka in our group have compared the results with SYTO-17 to results with the MTS assay, while recording changes in morphology at the light microscopic level which might reflect cytotoxic effects not evident from the dye permeability measurements or reductase activity assays. With few exceptions, the different assays for cytotoxicity are in good agreement, permitting us to rank the new CMTs in order of their cytotoxic activity towards LNCaP cells. In the course of undertaking the MTS measurements, we performed assays on cells maintained in RPMI medium containing phenol red and 4% fetal bovine serum as well as phenol red-free medium containing dextran- and charcoal-stripped serum in order to address concerns that the combination of serum and indicator dye might be exerting some hormone-like effects on the cells in culture. Indeed, we have found that cells maintained in the presence of phenol red and whole serum display consistently greater sensitivity to the cytotoxic CMTs than cells cultured in phenol red-free medium with charcoal/dextran-stripped serum. Observations of morphologic changes indicate that cytotoxicity is associated with a distinctive change in properties of the cells in culture: rather than maintaining a confluent layer of adherent cells, the LNCaP cells begin to round up, detach, and form clusters in the presence of the more cytotoxic CMTs, whereas the cells retain their shape and adherence in the presence of the noncytotoxic CMTs. Figures E.4 through E.6 illustrate the MTS assay results for CMTs 200, 300, and 303 which may be compared with the SYTO 17 results shown in the previous three figures, while Table E.2 summarizes the apparent IC_{50} values for the different CMTs after 48 hours of incubation with LNCaP cells in the presence and absence of phenol red, based on MTS assay results, along with descriptions of the morphologic changes which could be seen by light microscopy. It should be noted that changes in cell morphology including

rounding, detachment, and loss of confluence in other cell types, such as endothelial cells, are typically associated with loss of invasiveness.

To follow up on our initial observations on the selective cytotoxicity of CMT-300 towards prostate tumor cells but not towards normal prostate stromal cells, we have surveyed the effects of the newer CMTs on the R22 rat smooth muscle cells we routinely employ for elaboration of a stromal ECM. As explained below, we have temporarily suspended studies with human prostate stromal cells until our colleagues at Clonetics Corporation can supply us reliably with a suitable medium for culture of these cells under conditions which favor synthesis of a stromal ECM. The results Kimberly Guifoy in our laboratory has obtained with the R22 cells support our preliminary observations with human prostate stromal cells: only a very limited number of CMTs, including CMT-301, -302, and -303, but not CMT-300, show measurable cytotoxicity towards these normal stromal cells as evidenced by MTS reduction. However, among those CMTs which do not cause outright loss of reductase activity in the MTS assay, several (including CMT-300) cause morphologic changes such as rounding and loss of confluence at higher but still subcytotoxic doses. The results for cytotoxicity of CMTs towards R22 rat stromal cells are summarized in Table E.3. It is too early for us to assess whether the resistance of the rat cells to the CMTs will be shared by human stromal cells. We have only limited data for the sensitivity of human epidermal keratinocytes to the CMTs: these initial measurements suggest that these epithelial cells are more sensitive than the stromal cells we have examined, although the apparent IC_{50} values for the human keratinocytes are consistently higher than the corresponding values for LNCaP cells cultured under the same conditions. Our efforts will be directed during the coming year to evaluating the cytotoxicity of the CMTs towards normal human prostate epithelial cells.

F. Normal and Tumor-Derived Prostate Cell Culture Studies

As presented in the previous sections, we have made most extensive use of the LNCaP line of human prostate tumor-derived cells, which we routinely culture in RPMI medium supplemented with 4% fetal bovine serum [8]. We have already presented data that elimination of phenol red from the RPMI combined with pre-adsorption of the serum with charcoal and dextran reduces the sensitivity of the LNCaP cells to the cytotoxic effects of the CMTs. It has already been reported that such measures also reduce the expression of PSA by LNCaP cells [9], a phenomenon which is also of interest to us as PSA is a serine proteinase, the activity and expression of which might conceivably be modified by CMTs (discussed further below). While the elimination of phenol red and the use of "stripped" serum have been proposed as ways of reducing the hormonal influences on the cultured cells, a number of other components such as vitamins are also depleted by stripping of serum and we do not know the mechanism for the effects of culture conditions on our cytotoxicity assays. The use of phenol red-free medium with stripped serum is restricted to incubations with CMTs and is not employed for maintenance or expansion of cultures.

The LNCaP line was developed from a lymph node metastasis, while DU-145 and PC-3 cells were derived from brain and bone metastases respectively. The fact that these lines were derived from tumors which had already metastasized may be a significant reason for the success researchers had in maintaining and propagating them *in vitro*. The more typical experience of previous investigators is that "...lines are difficult to establish from explants of prostate tissue" [10]. To date Elizabeth Roemer has carried out three explant cultures of freshly isolated prostate tumor tissue obtained from surgical resections performed by Dr. S. Ali Khan. She first soaks the tissue specimens in RPMI containing gentamycin for at least 15 minutes prior to dicing them into < 1 mm pieces an transferring them to medium in T flasks. After a period of 4 to 9 days, outgrowth of cells can be

10

observed from about 5-10% of the tissue fragments. While the morphology of these cells is consistent with that of tumor rather than fibroblasts from the surrounding stroma, Ms. Roemer has not been able to carry the cells beyond a fourth passage. The cells do not appear to survive plating in 96 well plates, so we will have to design our studies with these explant cultures carefully to obtain maximum information from the limited material available.

Ms. Roemer has also been making progress in culturing normal human prostate epithelial cells for cytotoxicity testing with CMTs. These cells are obtained from Clonetics Corporation and are maintained in a specialized medium prepared by Clonetics under the supervision of their R&D Director, Dr. Soverin Karmiol. It is conceivable that, similar to the LNCaP cells, the normal prostate epithelial cells will also show some responsiveness to testosterone, but development of conditions to achieve maximum hormone sensitivity of cultured normal prostate epithelial cells *in vitro* is an objective outside the scope of this project.

Normal human prostate stromal cells have also been provided by Clonetics Corporation, but the serum-free medium which Clonetics has been endeavoring to develop for these cells has not been available on a regular basis, and may not be available reliably for several months. As a result of the erratic availability of stromal cell culture medium, we have not focused on preparation of a prostate-derived interstitial ECM from the cultured stromal cells, and have directed most of our efforts towards studies which can be carried out with prostate tumor-derived cell lines. Ms. Roemer's preliminary studies indicate that the normal stromal cells produce an ECM which is considerably richer in trypsin-sensitive components and poorer in collagen than the R22 rat smooth muscle cell-derived ECM and is much more fragile to routine handling during the steps of stromal cell lysis and washing. Projected modifications proposed by Clonetics for new formulations of their stromal cell culture medium may result in enhanced collagen synthesis by the confluent cells. We will quantitate matrix collagen synthesis by these cells using a commercially available ELISA for the soluble C-terminal propeptide which is released from procollagen as it is processed for incorporation into type I collagen fibrils. Regardless of our progress in generating prostate-derived stromal ECM, it should still be possible to employ the normal prostate stromal cells for evaluation of cytotoxicity of the different CMTs, using Clonetics' standard fibroblast medium for maintenance of the cells in culture. This medium is not suitable for supporting ECM synthesis, but does support maintenance of stromal cell viability.

G. Key Research Accomplishments
- Screened new CMTs for phototoxicity *in vitro* and identified several new compounds with phototoxicity no greater than that of doxycycline and less than that of CMT-300.
- Screened new CMTs for inhibition of human neutrophil elastase activity and for inhibition of collagenolytic and gelatinolytic activities of MMP-8 and MMP-9 respectively and identified compounds with inhibitory potency comparable to that of CMT-300.
- Screened new CMTs for cytotoxicity towards tumor cell lines and normal cells in culture and identified several new compounds with selective or preferential cytotoxicity towards at least one prostate tumor cell line (LNCaP).
- Developed procedures for culturing explants of resected human prostate tumors and achieved outgrowth of cells from the explants. These efforts are in early stages but are proceeding well.
- Progressed on developing culture conditions for normal human prostate epithelial and stromal cells for use as targets in cytotoxicity assays and for synthesis of interstitial ECM. These efforts are still in early stages and have been slowed by limited availability of

appropriate culture media.

H. Reportable Outcomes

The most tangible outcome of this project is projected to be the identification of one or more of the most promising new CMTs for inclusion in the drug development program of CollaGenex Pharmaceuticals and for evaluation in clinical trials. At this time, we believe it is premature for us to identify any one of the new CMTs as so unquestionably superior to the others as to merit such commitment of resources. However, several of the new CMTs appear to overcome the primary complication of undesirable photoreactions encountered in clinical trials with CMT-300, so that significant progress towards this outcome should be achieved within the time frame of the project.

More immediate outcomes are reflected in presentations at the 1999 Annual Meeting of the Society for In Vitro Biology. These presentations took the form of posters as well as an oral presentation. These presentations are listed below:

1. Zerler, B., E.J.Roemer, H.Raabe, A.Sizemore, and J.Harbell. (1999) Evaluation of the phototoxic potential of chemically modified tetracyclines using the 3T3 neutral red assay. *In Vitro* Cell. and Devel. Biol. in press
2. Simon, S.R., E.J.Roemer, W.Bellucci, Y.Gu, Q.L.Ying, C.Mannino, and E.Spero. (1999) Novel inhibitors in inflammation and Metastasis. *In Vitro* Cell. and Devel. Biol. 35:2A.
3. Bellucci, W.A., E.J. Roemer, S.R. Simon. (1999) A new screening assay for the evaluation of inhibitors of matrix metalloproteinases. *In Vitro* Cell. and Devel. Biol. 35:29A.
4. Boumakis, S., S.R.Simon and E.J.Roemer. (1999) Evaluation of the cytotoxicity of chemically modified tetracyclines (CMTs) on LNCaP prostate tumor cells. *In Vitro* Cell. and Devel. Biol. 35:31A.
5. Musacchia, L.C., Y.Gu, E.J.Roemer and S.R.Simon. (1999) Effects of chemically modified tetracyclines (CMTs) on colon and breast cancer cells *in vitro*. *In Vitro* Cell. and Devel. Biol. 35:31A.
6. Sehgal,B., S.R.Simon and E.J.Roemer. (1999) Chemically modified tetracycline 300 and its derivatives inhibit extracellular matrix degradation by polymorphonuclear leukocytes and Cystic Fibrosis sputum. *In Vitro* Cell. and Devel. Biol. 35:31A.
7. E.F.Spero, E.J.Roemer, S.R.Simon and C.Ren. (1999) The effect of chemically modified tetracyclines on the inhibition of sputum elastase in the presence of polyanions. *In Vitro* Cell. and Devel. Biol. 35:31A

I. Conclusions and Implications for Broader Project Goals

We may summarize our progress on the work outlined in our original proposal as follows:
1. We have evaluated a number of new CMTs provided by CollaGenex Pharmaceuticals, Inc. as potential "leads" for use in management of prostate cancer. Prior to these studies, no information regarding the biological activities of these new CMTs has been available. We have based our evaluations on the criteria of significant antiproteinase activity towards MMPs and the serine proteinase neutrophil elastase, selective cytotoxicity towards at least one prostate tumor cell line but not towards normal cells at comparable doses, and low phototoxicity following UV-A exposure. In addition to these criteria, Nungavaram Ramamurthy and Mark Bookbinder in Dr. Golub's group have also determined the pharmacokinetics of these new CMTs after oral administration to rats and Elizabeth Roemer in our group has evaluated their solubility in cell culture media. On the basis of all these criteria, we have identified six new CMTs, four of which are derivatives of CMT-300

(formerly CMT-3) and two of which are derivatives of CMT-310 (formerly CMT-10). The relevant properties of all these CMTs are summarized in Table I.1. None of these six compounds has less oral availability than the antimicrobial agents minocycline, doxycycline, or tetracycline, and none precipitates out of culture medium at doses which produce the desired biological responses. The development and implementation of criteria for selection of these derivatives for further study has also been summarized in a presentation at the 1999 Annual Meeting of the Society for In Vitro Biology [11]. On the basis of the studies reported in this Progress Report, CollaGenex Pharmaceuticals will provide samples of at least one of these new CMTs to Dr. Bal Lokeshwar at the University of Miami for further evaluation of their capacity to inhibit invasiveness of prostate tumor cells *in vitro*.

2. We have obtained evidence that the new CMTs have general properties which identify them as potential anti-metastatic drugs even beyond their specific use for management of prostate cancer. These properties include effects on the shape and growth characteristics of cells which may compromise their invasive potential and the capacity to downregulate levels of matrix metalloproteinases released by the cells into their surrounding environment. These additional properties further complement the selective cytotoxicity and antiproteolytic activity which we had originally observed for CMT-300 and which prompted us to propose its use in management of prostate cancer.

3. On the basis of recent results of clinical trials using CMT-300, we believe that phototoxicity associated with this compound may be sufficiently problematic to restrict its future use to relatively acute or short term (< 2 weeks) applications. Accordingly, we have ensured that the new CMTs we are continuing to investigate are all no more phototoxic than doxycycline, a tetracycline which has been administered on a long term basis to patients with acceptable levels of adverse side effects.

4. At the end of this first year, we have achieved outgrowth of tumor cells from a limited number of resected prostate cancers, and we have maintained normal human prostate stromal and epithelial cells in culture. However, we have not yet succeeded in preparing quantities of interstitial ECM from normal prostate stromal cells. We are positioned to evaluate cytotoxicity of the new CMTs towards prostate stromal cells and, hopefully, towards normal prostate epithelial cells. We anticipate that demonstrations of effects of the new CMTs on explants of human prostate tumors will be restricted to limited dose ranges which will be based on results with established tumor-derived cell lines. Effects on explants which we should be able to show would include cytotoxicity, such morphologic changes as rounding, clumping, detachment, and failure of the cells which grow out of the explants to achieve confluence, and possibly reduction in levels of proteinases released into the culture medium (at subcytotoxic doses). More quantitative evaluation of these effects of the new CMTs, including more rigorous determinations of dose dependence, may be confined to studies with established prostate tumor cell lines.

5. The evaluative criteria we are applying to characterize the new CMTs are intended specifically to identify a limited number of compounds for further consideration as candidates in the drug development program being undertaken by CollaGenex Pharmaceuticals for treatment of cancer. By concentrating our efforts during the coming year on six compounds which we have shown to possess the combined properties of low photoxicity, good antiproteinase activity, and selective cytotoxicity, we believe that the objectives of this project are being addressed.

J. References

[1]   Zerler, B., E.J.Roemer, H.Raabe, A.Sizemore, and J.Harbell. (1999) Evaluation of the phototoxic potential of chemically modified tetracyclines using the 3T3 neutral red assay. *In Vitro* Cell. and Devel. Biol. in press

[2]   Spero, E.F., E.J.Roemer, S.R.Simon and C.Ren. (1999) The effect of chemically modified tetracyclines on the inhibition of sputum elastase in the presence of polyanions. *In Vitro* Cell. and Devel. Biol. 35:31A

[3]   Sehgal, B., S.R.Simon and E.J.Roemer. (1999) Chemically modified tetracycline 300 and its derivatives inhibit extracellular matrix degradation by polymorphonuclear leukocytes and Cystic Fibrosis sputum. *In Vitro* Cell. and Devel. Biol. 35:31A.

[4]   Verheijen, J.H., N.M.Nieuwenbroek, B.Beekman, R.Hanemaaijer, H.W.Verspaget, H.K.Ronday, and A.H.Bakker (1997) Modified proenzymes as artificial substrates for proteolytic enzymes: colorimetric assay of bacterial collagenase and matrix metalloproteinase activity using modified pro-urokinase. Biochem. J. 323:603-609.

[5]   Bellucci, W.A., E.J. Roemer, S.R.Simon. A new screening assay for the evaluation of inhibitors of matrix metalloproteinases. *In Vitro* Cell. and Devel. Biol. 35:29A.

[6]   Musacchia, L.C., Y.Gu, E.J.Roemer and S.R.Simon. (1999) Effects of chemically modified tetracyclines (CMTs) on colon and breast cancer cells *in vitro*. *In Vitro* Cell. and Devel. Biol. 35:31A.

[7]   Boumakis, S., S.R.Simon and E.J.Roemer. (1999) Evaluation of the cytotoxicity of chemically modified tetracyclines (CMTs) on LNCaP prostate tumor cells. *In Vitro* Cell. and Devel. Biol. 35:31A.

[8]   Horoszewicz, J.S., S.S.Leong, E.Kawinski, J.P.Karr, H.Rosenthal, T.M.Chu, E.A.Mirand, and G.P.Murphy (1983) LNCaP model of human prostatic carcinoma. Cancer Res. 43:1809-1818.

[9]   Lee, C., D.M.Sutkowski, J.A.Sensibar, D.Zelner, I.Kim, I.Amsel, N.Shaw, G.S.Prins, and J.M.Kozlowski. (1995) Regulation of proliferation and production of prostate-specific antigen in androgen-sensitivie prostatic cancer cells, LNCaP, by dihydrotestosterone. Endocrinology 136:796-803.

[10]   Sramkowski, S.M., T.G.Pretlow, J.M.Giaconia, T.P.Pretlow, S.Schwartz, M.S.Sy, S.R. Marengo, J.S.Rhim, D.Zhang, and J.W.Jacobberger (1999) A new human prostate carcinoma cell line, 22Rv1. *In Vitro* Cell. and Devel. Biol.-Animal 35:403-409.

[11]   Simon, S.R., E.J.Roemer, W.Bellucci, Y.Gu, Q.L.Ying, C.Mannino, and E.Spero. (1999) Novel inhibitors in inflammation and metastasis. *In Vitro* Cell. and Devel. Biol. 35:2A.

Cytotoxic Effects of CMT 200 on LNCaP Cells
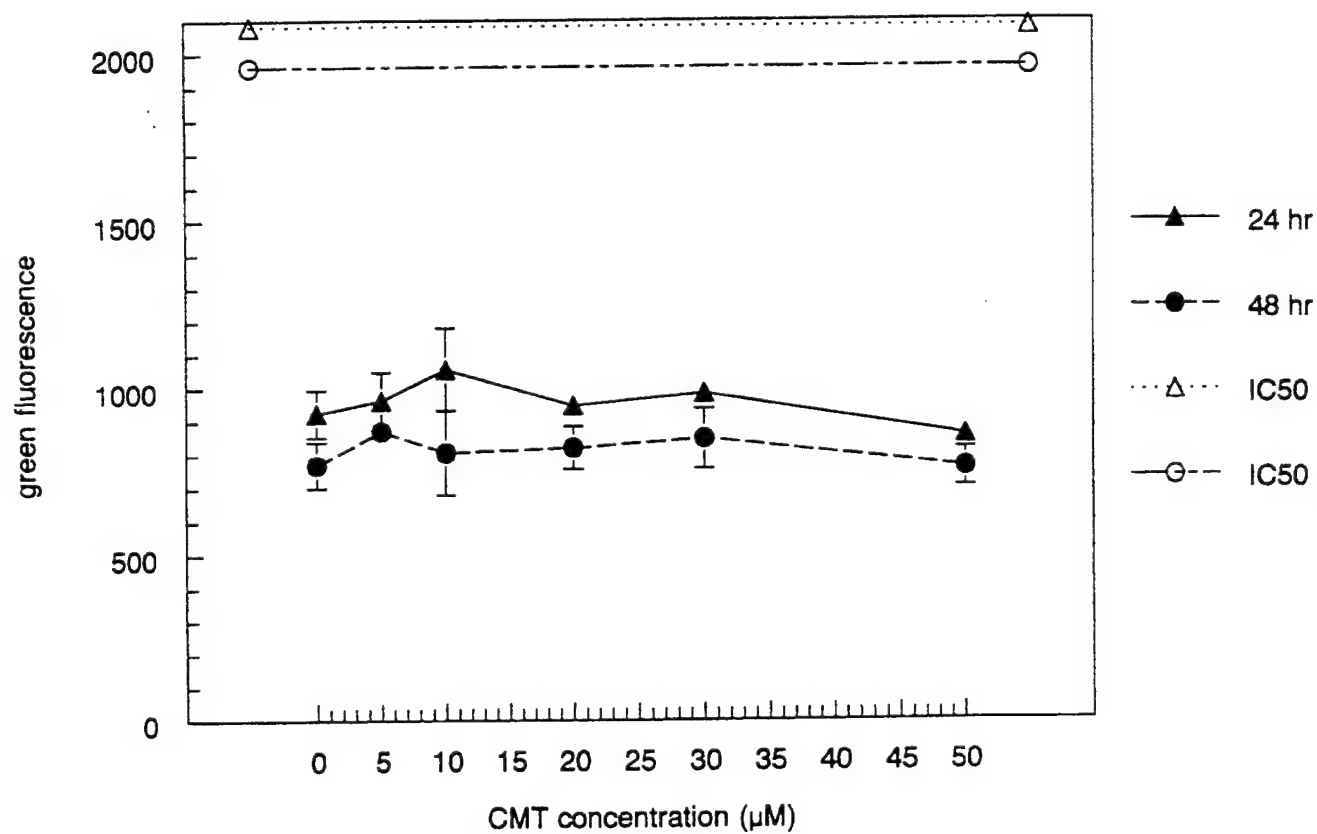
Cytotoxic Effects of CMT 300 on LNCaP Cells
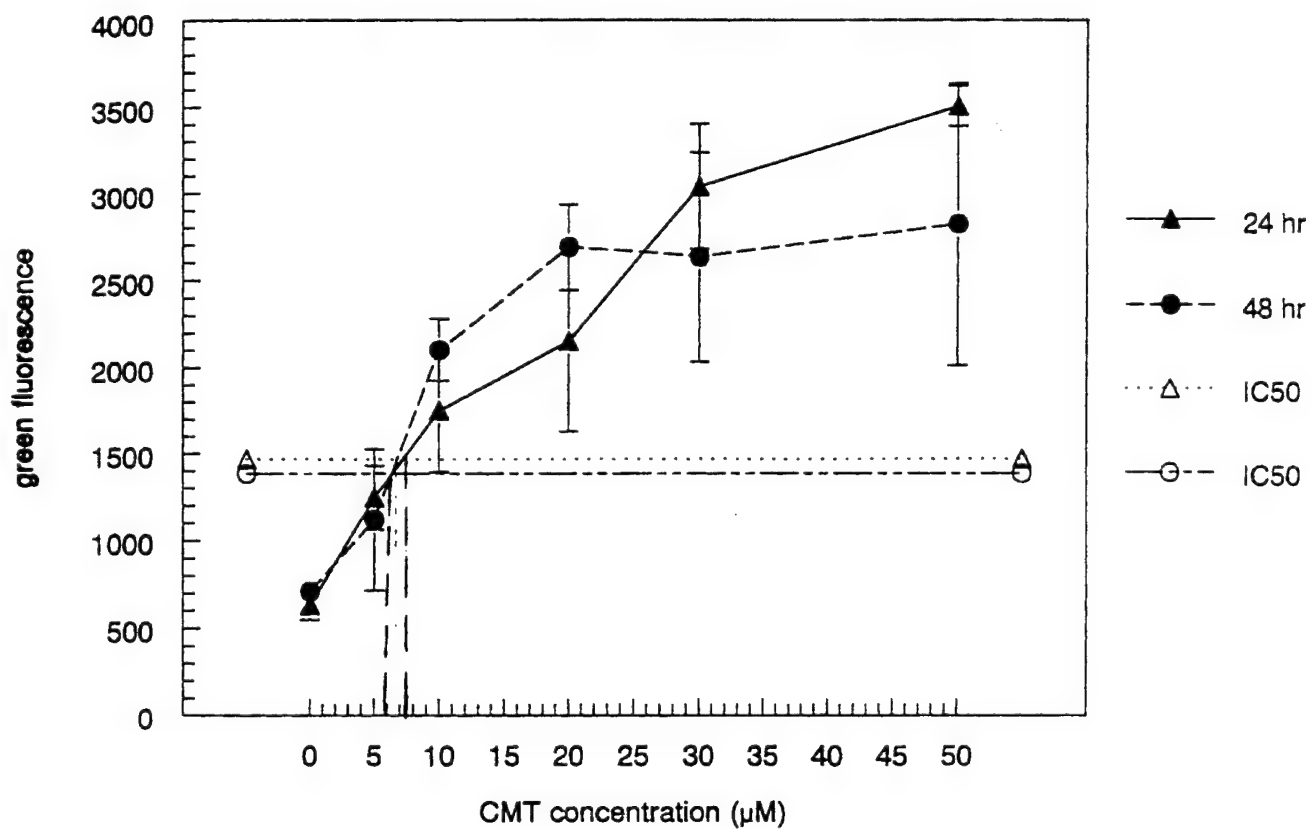
Cytotoxic Effects of CMT 303 on LNCaP Cells
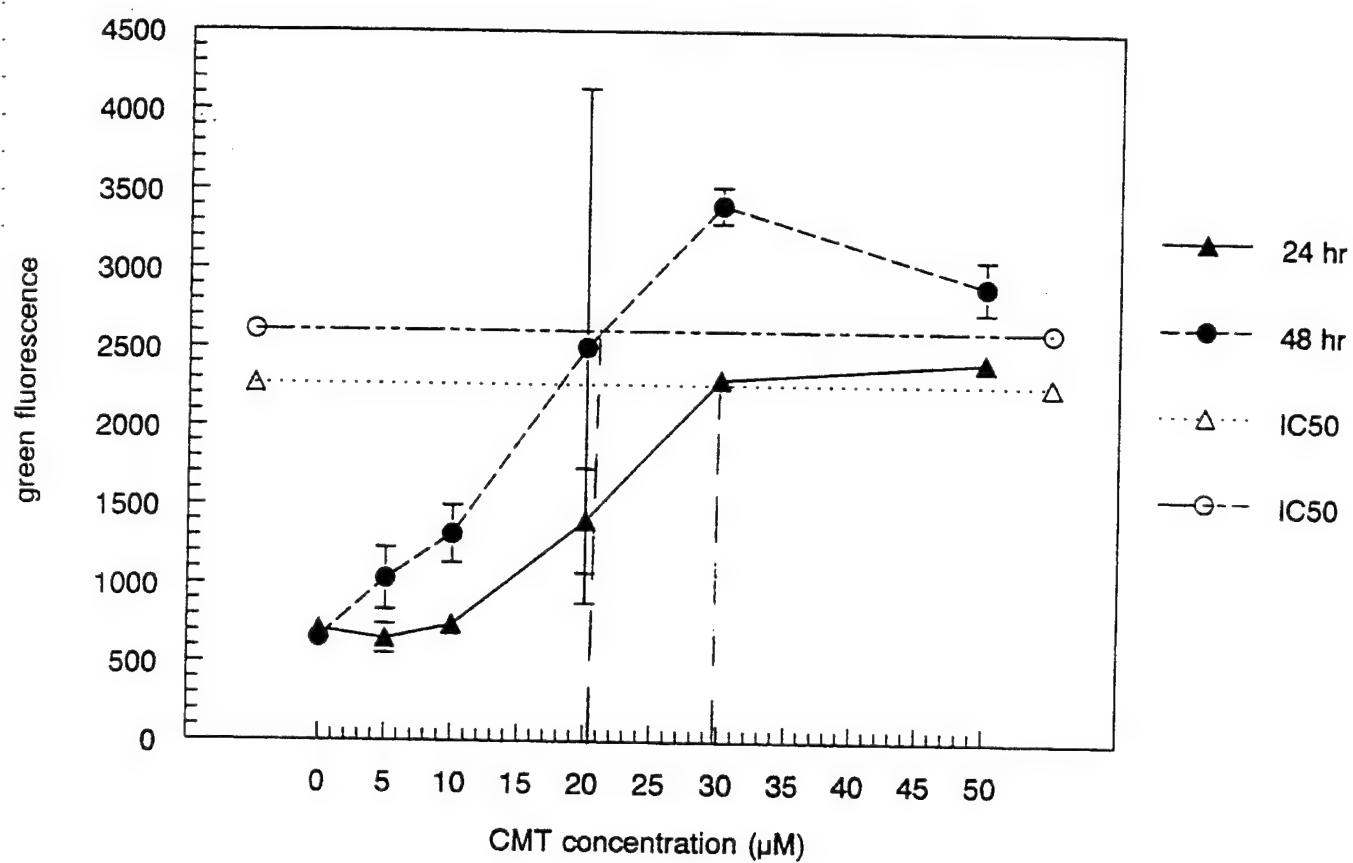
Figure E.4

LNCaP with CMT 200 (PR+/PR-)
After 48 hours incubation (8/20/99)

Legend:
- pr+ — △
- pr- — ○
- IC50+ — ▲
- IC50- — ●

Cytotoxicity determined with MTS assay; PR+ = medium contains phenol red and whole FBS
PR- = medium contains no phenol red and "stripped" serum

18

Figure E.5

LNCaP with CMT 300 (PR+/PR-)
After 48 hours incubation (8/20/99)

pr+ —△—   pr- —⊖—   IC50 —— IC50 —+—
                          ●   25 pr-
                        9 pr+
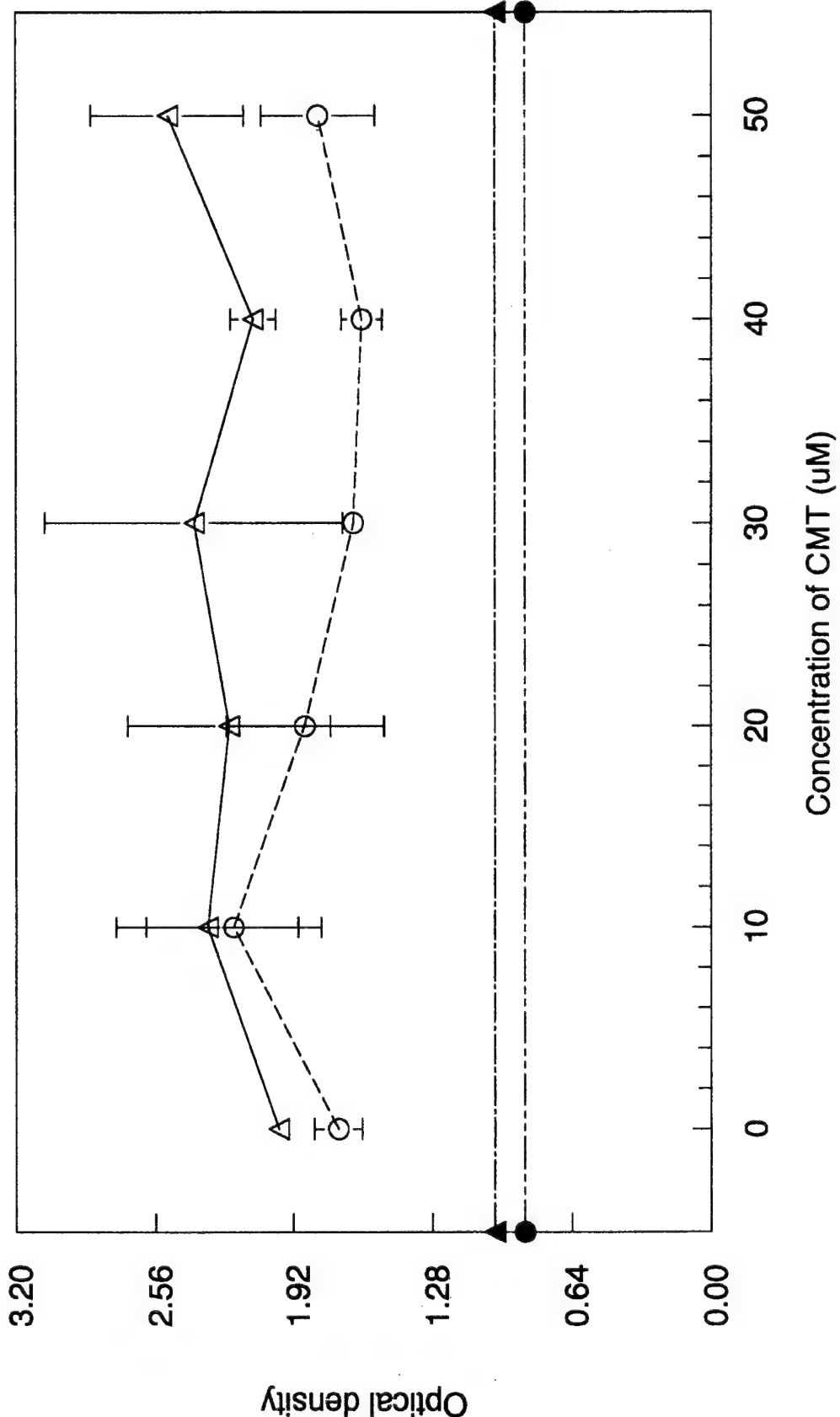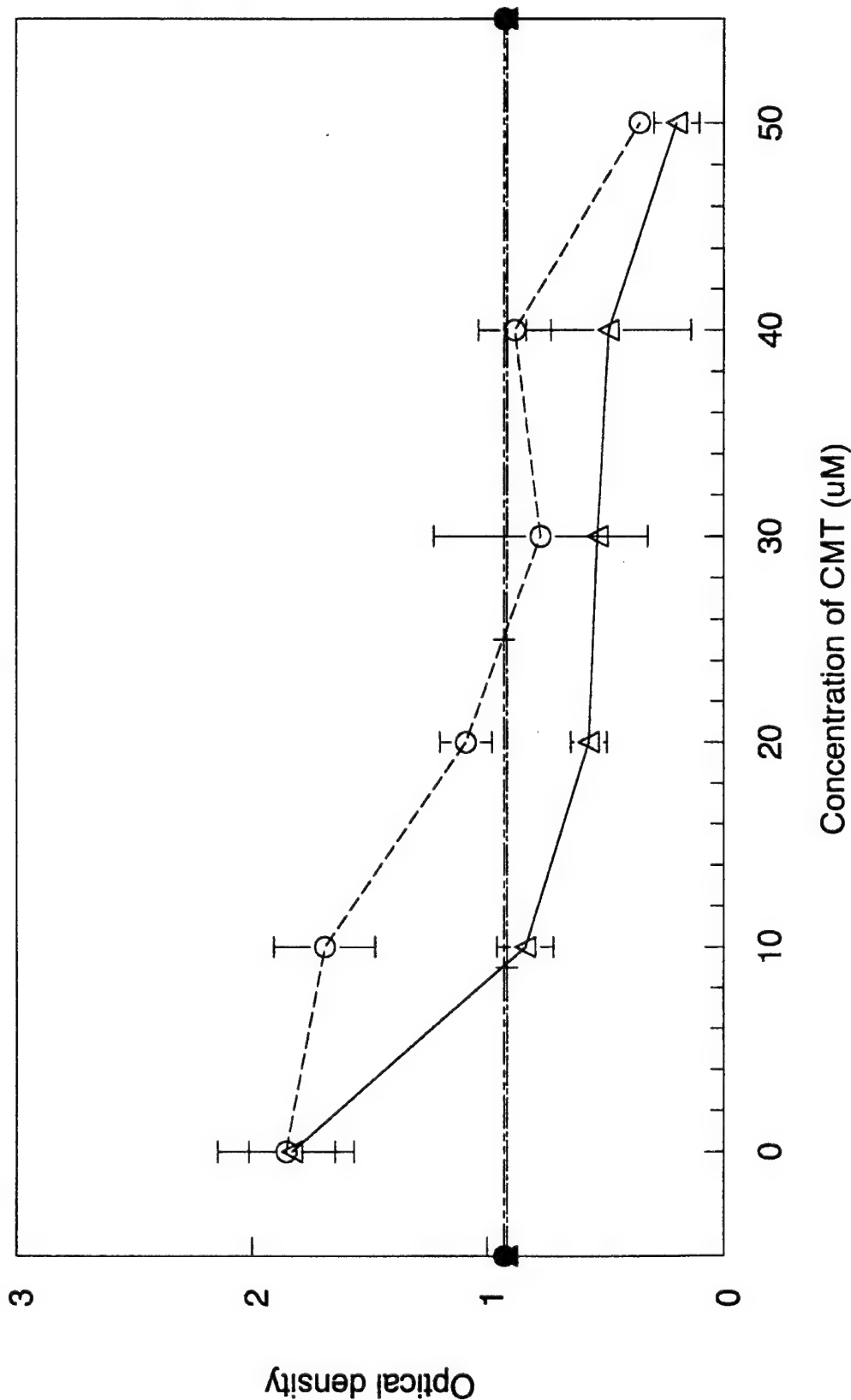
Concentration of CMT (uM)

Optical density

3   2   1   0

0   10   20   30   40   50

Cytotoxicity determined with MTS assay; PR+ = medium contains phenol red and whole FBS
PR- = medium contains no phenol red and "stripped"
serum

Figure E.6

LNCaP with CMT 303 (PR+/PR-)
After 48 hours incubation (7/9/99)
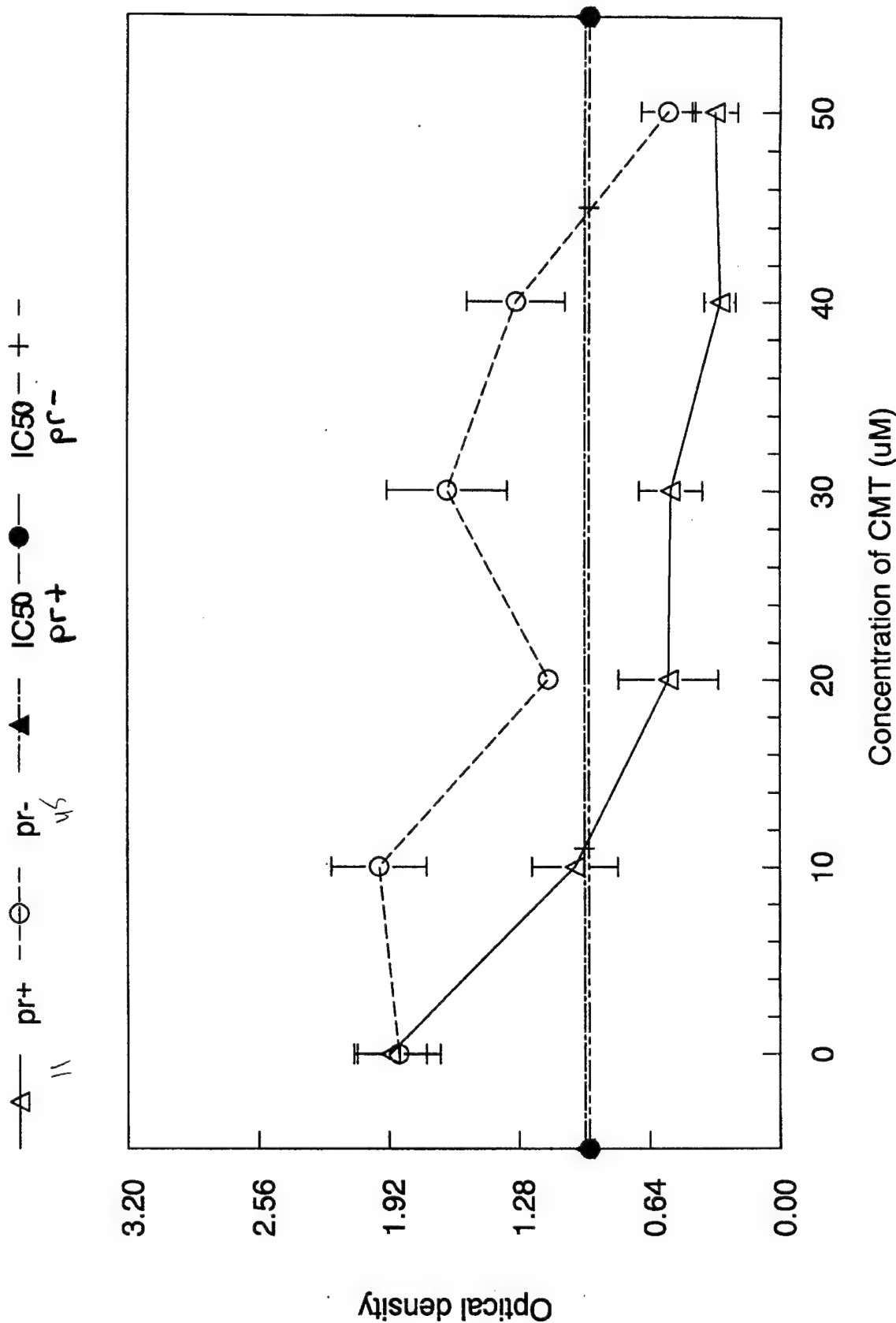
Cytotoxicity determined with MTS assay; PR+ = medium contains phenol red and whole FBS
PR- = medium contains phenol red and "stripped" serum

Rough ranking of CMTs by phototoxic potential
(Better Than Doxycycline)

CMT		phototox					
		Rank	MPE	PIF	IC50 ug/ml		
					+UV	-UV	
						ug/ml	rank
1001		1	0	1.0	>100	>100	nt
805	(308)	2	0.01	1.0	>100	>100	nt
307		3	0.017	1.321	82.3	90.467	2
804	(303)	4	0.0225	1.0	>100	>100	nt
311		5	.0275	-	>100	>100	nt
306	(806)	6	.0323	1.059	75.4	66.33	7
200		7	0.0437	1.0	>100	>100	nt
1002		8	.051 0.1497	1.122 1.0813	35.8 57.2	38.78 59.1867	11
308	(805)	9	.0823	1.0	>100	>100	nt
mino		10	0.0952	1.0	80.22	>100	nt
phototoxic (MPE)							
801	(305)	11	0.137	1.0	>100	>100	nt
802	(309)	12	0.1385	1.0	>100	>100	nt
310		13	0.115	1.547	36.68	61.67	8
803*		14	1.072	1.635	64.95	>100	nt
500		15	0.4545	3.001	35.585	>100	nt
600		16	0.4537	3.4587	30.097	>100	nt
309	(802)	17	0.379	4.905	43.15	82.433	4
phototoxic (PIF)							
303	(804)	18	0.543 0.5154	16.8127 13.9845	5.313 9.288	75.767 81.536	5
tetra		19	0.679	-	18.8	>100	nt
305		20	0.782	17.67	5.847	>100	nt
doxy		21	.8385	23.35	3.705	86.55	3
315		22	0.427 0.2907	24.089 16.3927	4.75 36.5	67.65 78.43	6

Rough ranking of CMTs by phototoxic potential: *(Worse than Doxycycline)*

CMT	phototox					
	Rank	*MPE*	*PIF*	*IC50*		
				+UV	*-UV*	
			.		*ug/ml*	*rank*
314	23	1.062 0.779	31.242 58.679	5.16 3.475	>100 68.83	nt
chlor		0.7299	41.0129	1.1959	26.1214	13
807	24	0.889	41.66	2.53	>100	nt
302*	25	0.408	45.883	1.003	51.147	9
808	26	0.842	46.8695	2.16	>100	nt
809	27	0.7766	55.222	1.9	>100	nt
313	28	0.314 0.5037	66.1365 59.75	0.394 0.9827	30.275 53.517	12
312	29	0.321 0.5353	76.5795 65.5247	0.509 1.116	41.585 61.0567	10
700	30	0.8957	222.462	.490	>100	nt
800	31	0.893	256.786	.5554	99.1	1
400*	32	0.7426	480.025	0.297	>100	nt
300	33	0.9233	819.580	0.2277	>100	nt
900	34	0.655	852.055	0.1086	>100	nt

Phototox will not be done: (no compound available)

CMT	Ptox		
	Rank		
301	**		
304	**		
806	**		

*: rank not yet clear, awaiting further data
nt= not toxic

Table C.1

K_i Values for Derivatives of CMT-3
(CMT-8 and most derivatives are inactive)

1. Sputum Elastase Activity (not purified)

CMT-3	44 µM
CMT-301	111 µM
CMT-302	11µM
CMT-303	10 µM
CMT-304	54 µM
CMT-305	104 µM (determined at low concentrations only)
CMT-306	77 µM
CMT-307	64 µM
CMT-308	49 µM
CMT-309	190 µM
CMT-310	65 µM

2. Sputum + 1% Albumin

CMT-3	152 µM
CMT-302	42 µM
CMT-303	44 µM

3. Neutrophil Elastase (purified)

CMT-3	43 µM
CMT-301	126 µM
CMT-302	12 µM
CMT-303	10 µM
CMT-304	167 µM
CMT- 305	(determination unreliable - insoluble at higher concentrations)
CMT-306	75 µM
CMT-307	97 µM
CMT-308	49 µM
CMT-309	166 µM
CMT-310	108 µM

4. Pancreatic Elastase

CMT-302	125 µM
CMT-303	166 µM
CMT-307	>300 µM
CMT-308	>500 µM

5. (Inactive against trypsin and chymotrypsin)

Table C.2

RANK by uM IC50

	Inhibition of PMN Mediated ECM Degradation						Inhibition of CF Sputum Mediated ECM Degradation		
	Run						Run		
CMT	1	2	3	4	5	6	1	2	CMT
1002	85				158				1002
302			160			115		100	302
303	112		165					100	303
313				200					313
310	270	158			232				310
314				225					314
300	165	218	230	350			260		300
308		300							308
311						315			311
315				322					315
301			325					280	301
1001	310				>400				1001
304			>400					>400	304
305		>400					>400		305
306		>400					>400		306
307		>400							307
309		>400							309
312				>400					312

Table D.1

uM

CMT		Cytotox (PT) Rank*	PT IC50 uM	PK:(rat) Cmax uM	Sputum elastase KI, uM	MMP-9 IC-50 uM	MMP-8 IC-50 uM
1001		nt	>200		8	30	
805	(308)	nt	>200	2.56	na	38.5	
307		2	164.6	3.02	64	55	
804	(303)	nt	>200	4.2	na	na	
311		nt	>200	***	5.5	insol.	
306	(806)	7	150.8	2.26	77	69	
200		nt	>200	0.5			
1002		11	71.6	0.48	13	49.5	10
308	(805)	nt	>200	7.1	49	15	1.5
mino		nt	160.4			na	
phototoxic (MPE)							
801	(305)	nt	>200	0.9	na	na	
802	(309)	nt	>200		na	na	
310		8	93.4	19.4	65	110	50
803*		nt	129.9		na	na	
500		nt	71.2				
600		nt	60.2	0.54			
309	(802)	4	86.3	1.52	190	na	
phototoxic (PIF)							
303	(804)	5	10.6	1.04	10	32	4
tetra		nt	37.6	0.48		na	
305		nt	11.6		104		
doxy		3	7.4			na	55
315		6	9.5	20	1.17	15	11
302		9	2	1.16	31.1	55.5	
800		1	1.1	37.7	na	na	
300		nt	0.45	6.66	44		4

* cytotoxicity from the no UV exposure of the phototox study, nt = not toxic, value based on all compounds, n=34, nt=22, so #1 is the 23rd compound.

CollaGenex

Controls and COL-1 - COL-10

LNCaP Toxicity: Syto 17

COMPOUND	IC50 (uM)	
	24hr	48hr
Doxy	—	43.0
Mino	ND	ND
Tetra	ND	ND
COL-1	—	9.0
COL-2	—	—
COL-3	7.5	6.0
COL-4	35.0	13.0
COL-5	—	—
COL-6	45.0	48.0
COL-7	Floorescent	
COL-8	37.0	25.0
COL-9	ND	ND
COL-10	—	—

COL-300 - COL-315

LNCaP Cytotoxicity: Syto 17

COMPOUND	IC50 (uM)	
	24hr	48hr
COL-300 (3)	7.5	6.0
COL-301	25.0	15.0
COL-302	34.2	23.5
COL-303	30.0	21.0
COL-304	—	40.0
COL-305	—	41.0
COL-306	33.0	25.5
COL-307	ND	ND
COL-308	—	—
COL-309	—	—
COL-310 (10)	—	—
COL-311	—	29.0
COL-312	5.0	3.0
COL-313	3.5	4.0
COL-314	11.0	4.0
COL-315	4.0	5.0
COL-1000 (10,310)	—	—
COL-1001	—	—
COL-1002	—	—

Visual observation on LNCaPs treated with CMTs

CMT	IC 50 (µM) PR+	PR–	Observations
100	16	48	All cells cluster together starting at 30 uM on PR+, more round cells presented on PR- starting at 30 uM, they cluster together at 50 uM.
200	nT	nT	CMTs show no effect on LNCaPs on both PR+ and PR- (95% confluence)
300	9	25	Cells cluster together starting at 10 uM, all clustered at 30 uM, 40 uM and 50 uM on both PR+ and PR-
301	8	17.5	Lots of cells cluster together at 10 uM. No lives at 20 uM and beyond. (same in both PR+ and PR-)
302	7.5	10	Round cells show up at 10 uM. Starting at 20 uM up to 50 uM, all cells cluster together.
303	11	45	A lot of round cells starting at 10 uM (50%), at 40 and 50 uMs, all cells clump together.
304	nT	nT	CMTs show no effect on wells (80% confluence), slight showing up of round cells on 30 uM, 40 uM, and 50 uM.
305	17	48	Some round cells built up at 20 uM. At 40 uM and 50 uM, about 40% of round cells present.
306	11.5	43	Some round cells built up at 10 and 20 uM (20-30%). At 40 and 50 uMs, all cells clump together.
307	42	NT	Round cells built up at 50 uM on PR+
308	NT	NT	80% confluent. Round and fiber shaped cells mixed throughout all concentration of CMTs.
309	~66.5	~75	CMTs show no effect on LNCaPs on both PR+ and PR- (90% confluence)
310	37	50	Cells cluster together at 20 uM, but not all cluster even at 50 uM (PR+ and PR-)
312	nT	NT	CMTs show no effect on LNCaPs on both PR+ and PR-
313	NT	NT	CMTs show no effect on LNCaPs on both PR+ and PR-
311	NT	NT	

IC 50(μM)

	PR+	PR-	
314	NT	NT	CMTs show no effect on LNCaPs on both PR+ and PR-
315	NT	NT	CMTs show no effect on LNCaPs on both PR+ and PR-
400	9.3	38	Cells cluster together starting at 10 uM, all clustered at 30, 40, and 50 uMs on both PR+ and PR-
500	NT	NT	CMTs show no effect on LNCaPs on both PR+ and PR-
600	48	NT	CMTs show no effect on LNCaPs on both PR+ and PR-
700	NT	NT	CMTs show no effect on LNCaPs on both PR+ and PR-
800	6	35	A lot of round cells on 10 uM and all clustered together beyond 10 uM.
801	NT	~77	Only little round cells built up starting at 30 uM. At 50 uM, it's about 10% of them present.
802	NT	NT	CMTs show no effect on LNCaPs on both PR+ and PR-
803	32	NT	Some round cells present at 30 uM on PR+ and cells cluster at 40 and 50 uMs.
804	NT	NT	CMTs show no effect on LNCaPs on both PR+ and PR-
805	NT	NT	CMTs show no effect on LNCaPs on both PR+ and PR-
807	NT	NT	CMTs seem not effective, but some round cells showed at 50 uM (90% confluence)
808	NT	NT	CMTs show no effect on LNCaPs on both PR+ and PR- (90% confluence)
809	42	51	Round cells show up minimally at 30 uM, cells cluster at 40 and 50
uMs.	37.5	NT	um
900	20	44	At 40 and 50 uM, LNCaPs has somewhat clustered together (20-30% clustered). Some round cells present at these concentration.
1001	NT	NT	CMTs show no effect on LNCaPs on both PR+ and PR-
1002	NT	NT	CMTs show no effect on LNCaPs on both PR+ and PR-

IC 50 (μm)

PR+	PR-		
46.5	~60	**Minocyclin**	On both PR+ and PR-, there is minimally clustering of LNCaPs at 50 uM.
27.5	49.5	**Doxycyclin**	LNCaPs start cluster together at 30 uM, more round cells at 30 uM. At 50 uM, 80% had clustered together.
NT	NT	**Erthromycin**	Very minimally effect on LNCaPs although some clustering showed on 40 and 50 uMs
NT	NT	**Tetracyclin**	CMTs show no effect on LNCaPs on both PR+ and PR-

All IC_{50} values determined using MTS assay

Note: PR+ = medium contains phenol red and 4% whole FBS

PR- = medium contains no phenol red and serum was been "stripped"

NT = no toxicity up to 100 uM

CMT	IC 50		Observations	
	Confluent	Sub-Confluent	Confluent	Sub-Confluent
301	8.6 um	32.8 um	20 R++	20:R++; LC
302	43.5 um	35 um	20 R++	20:R++
303	~ 52 um	~ 53 um	20 R++	20:R++
900	~60-65 um	NA	A:R= 20:PP	A:R=; 20:PP
310	NA	NA	50:R-	50:R-
801	NA	NA	A:R=	10:R=/50:R-
307	NA	NA	50:R-	A:R=
309	NA	NA	A:R=	A:R=; 50:LC
805	NA	NA	A:R=	A:R=; 50:LC
Mino	NA	NA	R=	NR
308	NA	NA	NR; P	NR; P
802	NA	NA	A:R=	A:R=
1002	NA	NA	10:R=/50:R-	10:R=/50:R-
1001	NA	NA	10:R=/50:R-	10:R=/50:R-
300	NA	NA	20:R;/50:R++; 50:LC	20:R+;/50:R++ 50:LC
315	NA	NA	10:R=/50:R- 30:PP	10:R=/50:R- 30:PP
305	NA	NA	10:R=/50:R-	40:R; LC
311	NA	NA	A:R=	??
803	NA	NA	50:R; 50:S; LC	50:R-; S; LC
Tetra	NA	NA	A:R=	10:R=/50:R-
312	NA	NA	??	A:R= 40:PP
313	NA	NA	10:R=/50:R-	A:R=; 50:LC
314	NA	NA	30:R- 30:PP	A:R=; 30:PP
700	NA	NA	10:R=/50:R- 50:PP	A:R=; 30:PP

| CMT | IC 50 | | Observations | |
	Confluent	Sub-Confluent	Confluent	Sub-Confluent
800	NA	NA	R++	R++
807	NA	NA	10:R=/50:R-	A:R=
808	NA	NA	A:R=	A:R=
809	NA	NA	10:R=/50:R-	A:R= 50 LC
100	NA	NA	A:R=	A:R= LC
304	NA	NA	A:R=	A:R=
400	NA	NA	50:R-	A:LC
200	NA	NA	10:R=/50:R-	10:R=/50:R-
500	NA	NA	10:R=/50:R-	10:R=/50:R-
600	NA	NA	A:R=	A:R=
Doxy	NA	NA	10:R=/50:R-	10:R=/50:R-
804	NA	NA	A:R-	A:R=
306	NA	NA	NR	NR

R=	0-10% Rounding of Cells
R-	10-20% Rounding of Cells
R	25-50% Rounding of Cells
R+	50-75% Rounding of Cells
R++	75-100% Rounding of Cells
A:R=	Minimal increase in cell rounding as concentration increases
10:R=/50:R-	Slight increase in cell rounding as concentration increases
PP	Precipitate Seen
LC	Wells become less Confluent than Controls
10-50	Lowest concentration (um) at which change was seen
A	Change seen in all Concentrations
S	Cells become elongated
NA	No Cytotoxic Activity

Note: all IC_{50} values were determined with MTS assay

Table I.1

9-99, CollaGenex   **DATA: 6 lead compounds**

CMT		PK: (rat) ug/ml	PT uM	Cyto-Toxicity							Function		
				"Normal" Cells			Tumor Cells: uM						
				Cytotox (PT) *RANK*	NHEK 48h uM	R22 uM	LNCaP CyToX 48h	LNCaP MTS 48h		Colo 205 48h	elast. KI uM	MMP-9 uM	MMP-8 uM
								+PR	-PR				
1001		**	>200	n(>200)	(36)	n =/-	n	n	n	n	8	30	23
306	(806)	1.13	150.8	30	(9)	n n 14	25.5	14	44	n	77	69	7
1002		0.24	71.6	34	(20)	n =/- n	n	n	n	n	13	49.5	11
308	(805)	3.6	>200	n(>200)	(14)	n n/P n	n	n	n	n	49	15	1.5
mino		0.15	160.4	n(>200)	65.4	n = n	**	46.5	60	n	**	n	12
310		9.69	93.4	31	**	n - **	n	37	50	**	65	110	50
303	(804)	0.52	10.6	28	6.74 (14)	52 ++ **	21	10.5	45	36	10	32	4
tetra		0.24	37.6	n(>200)	121.3	n = **	**	n	n	**	**	n	**
doxy		1	7.4	7.4	61.5	n =/- **	43	27.5	49.5	30 n	**	n	65
315		10	9.5	9.5	(100)	n =/-P n	5	n	n	n	1.17	15	10
800		15	1.1	23	82.7	n ++ **	25	6	35	90 n	n	n	**
300		3.33	0.45	n(>200)	13.5	n ++ 8	6	9	28	19 42	44	**	4

Cytox PT:cytotoxicity from the no UV exposure of the phototox study, maximum dose 200uM
**: no data, sample not done or not do-able
n: no effect, not toxic up to tested dose
(>XXX): highest concentration screened, any toxicity or effect not detected
(xx): value not confident, assay to be repeated
R22:   x   48 hr
         X: visual: -, =, +, ++: least to most effect, cell rounding and detachment, P: precipitate
         x   4 day

Table I.1 (continued)

9-99, CollaGenex   Rank and Score of 6 lead and 6 **reference** compounds

Rank	Total Score	CMT		PK: (rat)	PT	CytoToxicity: rank: (score)		Function	
						"Normal" Cells	Tumor Cells	Elast KI	MMP (9 + 8)
3	25	1001		8*	1	2: (8)	8: (22)	2	4: (7)
5	27	306	(806)	4	2	8: (18)	3: (13)	6	4: (7)
7	32	1002		6	4	7: (16)	8: (22)	3	4: (7)
1	19	308	(805)	3	1	2: (8)	8: (22)	4	1: (2)
8	33	*mino*		7	2	3: (9)	7: (21*)	8*	6: (10)
7	32	310		2	3	9: (19*)	6: (19)	5	7: (11)
5	27	303	(804)	5	6	10: (21)	2: (9)	2	2: (4)
10	38	*tetra*		6	5	1: (7)	9: (24*)	8*	9: (14*)
9	34	*doxy*		4	6	4: (10)	4: (14)	8*	8: (13)
2	20	315		2	6	3: (9)	5: (18)	1	3: (5)
6	31	800		1	7	5: (12)	2: (9)	7	9: (14*)
4	26	300		3	7	6: (15)	1: (5)	4	5: (9**)

*, or **no data, worst possible value assigned. please note: **: value was significantly different than data from assays of similar type, all others value was very similar, or no comparison was possible

JS-4

Novel Inhibitors in Inflammation and Metastasis. S.R.SIMON, E.J. Roemer, W. Bellucci, Y. Gu, Q.L. Ying, C. Mannino, and E. Spero. Pathology Dept, SUNY at Stony Brook, Stony Brook, NY 11794-8691. E-mail: SSIMON@PATH.SOM.SUNYSB.EDU.

Agents to inhibit destruction of connective tissue by inflammatory cells and invasive tumor cells should ideally target activities of multiple classes of proteinases. Endogenous antiproteinases with specificities against individual proteinases may show impressive potency when studied in isolation, but the synergistic actions of multiple proteinases, along with other components of the inflammatory response or the invasive phenotype, may inactivate these endogenous defenses. Inhibitors of multiple proteinases should be especially effective under these circumstances, blocking pathologically excessive proteolysis and protecting endogenous antiproteinases. We have been evaluating several chemically modified tetracyclines (CMTs) which possess pleiotropic inhibitory activities, using a series of in vitro assays in which the action of multiple proteinases can be monitored. A major tool in these assays has been the use of radiolabeled complete interstitial extracellular matrices as substrates for degradation by inflammatory cells, tumor cells, or their conditioned media. The most promsing of the CMTs inhibit matrix metalloproteinase (MMP) activity as well as activity of the serine proteinase neutrophil elastase (NE). Screening of inhibitory potencies employs a novel coupled amidolytic assay for MMP inhibition with prourokinase as an MMP substrate, and a dye-binding assay for elastinolysis in addition to the classical amidolytic assay for inhibition of NE activity. These assays are all adaptable to use with inflammatory or tumor cells as well as purified proteinases and can be modified to introduce components which are present in the microenvironment of acute inflammatory foci or invasive tumors and which may alter the effectiveness of synthetic antiproteinases. The distinct specificities of synthetic and endogenous antiproteinases are used in the assays to evaluate the capacity of synthetic inhibitors to protect endogenous antiproteolytic defenses. (Supported by NIDR [DE-10985], STRC, USAMRMC, Collagenex Pharm., and SUSB Biotechnology Center)

VT-1029

Evaluation of the Cytotoxicity of Chemically Modified Tetracyclines (CMT) on LNCaP Prostate Tumor Cells. S.Boumakis, S.R. Simon and E.J. Roemer, Department of Pathology, State University of New York at Stony Brook, Stony Brook, NY, 11794-8691

The major source of morbidity and mortality of prostate cancer is due to the invasive and metastatic potential of the tumor cells. Invasiveness may be linked to expression of one or more members of the family of matrix metalloproteinases (MMPs). Other proteinases, such as the serine proteinases, may also participate in tumor cell invasion and degradation of tissue stroma. We use LNCaP prostate tumor cells in in vitro assays to evaluate the capacity of chemically modified tetracyclines with multiple antiproteolytic activities to inhibit tumor-mediated tissue destruction. We have employed an in vitro system based upon a complete interstitial extracellular matrix (ECM) synthesized by rat heart smooth muscle cells (R22s), on which LNCaP cells can be cultured, to model tumor-mediated tissue degradation. Before undertaking studies on tumor cell-mediated matrix degradation, optimal LNCaP culture conditions were characterized. Preliminary studies determined which medium formulations, plating densities and feeding schedules best met LNCaP growth requirements, as well as the time required for the cells to reach confluence. LNCaP cells appear to be unusually sensitive to cytotoxic action of the CMTs, and may be useful targets for establishing whether this family of drugs may be selectively tumoricidal. Cytotoxicity assays which address this additional mode of CMT action against tumor cells help identify candidate compounds for further study. [funded by USAMRMC, Collagenex Corp, and NSF RAIRE Grant No. STI 9620074]